Primer

Circadian clocks

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The biological world is not static: organisms inhabit environments which change predictably over the course of the day and night. There will therefore be selective advantage to any individuals able to anticipate these changes and attune their physiology and behaviour accordingly. Anticipation of daily cycles is conferred by endogenous timers, biological clocks with an intrinsic period of approximately one day (hence *circa*dian).

It is now widely accepted that circadian timing is a fundamental property of life, and the ubiquitous expression of circadian rhythms has provided the biologist with many ways of looking at the clock. As a result, circadian timing now stands out as a paradigm for the explanation of how apparently complex patterns of behaviour and physiology might arise from the properties of a relatively small number of gene products, acting in single cells.

How rhythms are synchronized

In their natural environment, unicells, plants, fungi and animals display a range of metabolic, physiological and behavioural rhythms, each with a characteristic timing, or 'phase angle', relative to day and night. For example, our brains are 'turned on' and our musculo-skeletal performance is maximal during the day, whereas our physiology is geared to facilitate growth and bodily repair during nocturnal sleep. In higher plants the assembly of photosynthetic apparatus starts up in the hours before dawn, preparing the individual to take full advantage of the sunlight. Internal temporal order not only makes for effective adaptation to the environment; the temporal segregation of or potentially conflicting processes also makes for a more efficient biological machine.

These rhythms must be generated and maintained in some way. A critical observation is that when people, plants or fungi are isolated under constant environmental conditions, their rhythms do not stop or become disorganized. Most of them persist with a period that deviates only slightly from 24 hours and they are said to 'free-run'. The free-running period has two remarkable properties: it is very accurate (the deviation may be only minutes in 24 hours, and constant from cycle to cycle) and it is defended against changes in ambient temperature, in contrast to other metabolic functions.

To be effective, the biological clock — or circadian oscillator — needs to be synchronized or 'entrained' to the solar cycle, so that the rhythms it drives run at exactly 24 hours with a defined phase relationship to day and night. The principal entraining cue is light and darkness: bright light presented to free-running individuals held in dim light or darkness can reset the oscillator and so shift their rhythms. The direction and magnitude of the shift depend on when in the cycle light is encountered. During subjective day, that is, that part of the cycle when the subject behaves as if it were daytime, light may have little effect (this is called the 'dead zone'). In contrast, light delivered during the start of subjective night will delay the clock and its rhythms, whereas light presented later in subjective night advances them.

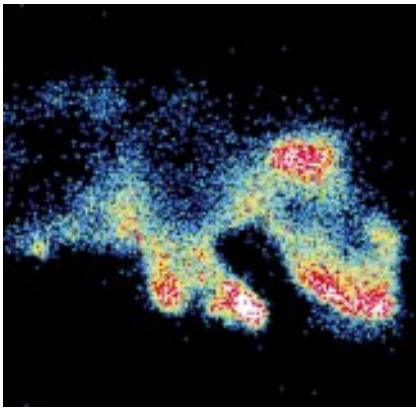
A plot of the phase shift as a function of when the pulse was presented yields a phase response curve (PRC). This varying response to a standard stimulus as a function of when in the cycle the stimulus is presented provides positive proof that the rhythms being examined are driven by a true endogenous oscillator rather than any other form of timing mechanism. The general shape of phase response curves to light is consistent across species, although differences in detail such as the amplitude of shifts and the extent of the dead-zone of subjective day vary between species and with experimental conditions. As a result, the phase response curve is the oscillator's finger-print.

Identifying clocks

A number of approaches have been used to localize circadian clocks: examining isolated tissues for spontaneous rhythms *in vitro*, testing the effects *in vivo* of local ablation and stimulation, and transplantation of putative clock tissues between subjects (see Figure 1 and blue box). Light is central to entrainment, and it is not surprising that in animals there is a close anatomical association between photoreceptors and clocks. In molluscs, flies, frogs and mammals, the eyes contain endogenous clocks, as does the pineal gland, the 'third eye' of some lower vertebrates.

But this list is far from comprehensive. It is likely that endogenous clocks regulate local

Figure 1

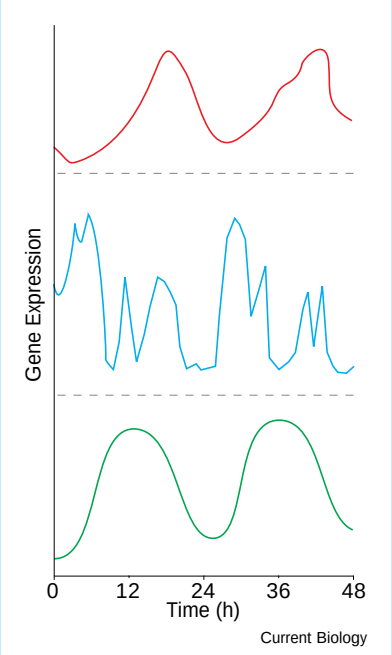


In this live mouse pup (shown nose towards bottom right), the circadian transcriptional programme has been imaged in real time, by means of the expression of a transgene in which the human c-*fos* promoter drives expression of a luciferase reporter gene. In this way, the circadian rhythm of c-*fos* expression in the clock nucleus of the suprachiasmatic nucleus (SCN) has been observed. The bioluminescence in this image results from c-*fos* expression in the skin. (For more details, see *Current Biology* 1997, 7:758–766.)

What goes up . . .

It is widely accepted that the core oscillation of many, if not all, circadian clocks is based on some form of auto-regulatory, transcriptional feedback loop. Modern imaging techniques now make it possible to view in real-time the *in vivo* circadian patterning of expression of putative clock genes and also the rhythms of expression of their effector, clock-controlled, genes which drive overt rhythms.

The key to these clocks lies in the protein–DNA interactions regulating promotor activity. By generating transgenic organisms in which rhythmically active promotor elements are hooked up to reporter sequences, core events of the clockwork can be observed, and with further mutagenesis the secrets of the clock promoters will be revealed.



The graph shows the rhythmic expression of clock genes, or clock-controlled genes, in three different organisms, in each case measured via the expression of a bioluminescent reporter gene. The blue line shows the expression of a *period* transgene in *Drosophila.* The red line shows the expression of a chlorophyll *a*/*b*-binding protein (*CAB*) reporter gene in *Arabidopsis*. The green line shows the expression of a photosystem II gene in cyanobacteria. (For more details, see: *Neuron* 1996, 16:687–692; *Science* 1995, 267:1161–1163; *Science* 1994, 266:1233–1236, respectively.)

circadian rhythms in many tissues of multicellular species, and in algae there is evidence of multiple clocks in single cells. In higher plants most tissues are light sensitive and clocks are local, distributed widely and co-ordinated loosely, if at all. For example, circadian rhythms of leaf movement which direct the photosynthetic surface optimally to incident light are driven by cells in the pulvinus at the base of the leaflet, whereas stomatal guard cells express a parallel but independent circadian rhythm of opening and closing both in the intact plant and when isolated *in vitro*. In fungi the entire mycelial mat can be rhythmic (see 'Biology in Pictures', page 669), but differences in free-running phase between the edge and centre indicate local control, with synchrony being imposed only when all areas experience the same light–dark cycle.

In mammals the principal pacemaker governing behavioural and endocrine rhythms lies within the suprachiasmatic nuclei (SCN) of the hypothalamus. The 10,000 or so neurons of each nucleus provide a remarkable example of localization of function within the nervous system. Not only does the nucleus itself display circadian rhythms of metabolic and electrical activity when isolated *in vitro*, circadian rhythms are also expressed by individual isolated SCN neurons.

The co-ordinated output of these cellular oscillators has a pervasive influence on almost all aspects of neural function, including the timing of sleep, the most fundamental change in brain state. The SCN is entrained to the light–dark cycle by direct retinal fibres, although additional neural inputs convey other stimuli, for example, social cues, which can reset our circadian timing. Knowledge gained about these resetting pathways should make it possible to develop therapies to alleviate or even correct the problems associated with clock dysfunction, not just jet-lag but the much bigger problems of sleep disturbance, especially in old age.

Clock mechanisms

What of the molecular genetic basis of the clockwork? Spectacular advances in this area have been made in the past decade, especially in the fruit fly *Drosophila* and the fungus *Neurospora*, for which a number of 'clock genes' have been identified: *frequency* (*frq*) in the fungus, and *period* (*per*) and *timeless* (*tim*) in the fly. The superficial similarities between the molecular clockwork of these contrasting species are striking. In both, the transcription of the genes displays a 24-hour rhythm under a light–dark cycle and free-runs in constant conditions.

The spontaneous rhythm is a consequence of negative feedback — a feature necessary for any self-sustaining oscillator — in these cases, it is exerted on the genes by their protein products. Some time after transcription is initiated, mRNA accumulates to be followed by new protein. Eventually the 'clock protein(s)' is able to gain access to the nucleus and in some way inhibit expression of the encoding gene(s). Consequently, mRNA levels and protein synthesis start to fall and degradation and/or inactivation of the existing protein allows transcription of the gene to re-commence. Autoregulatory feedback such as this is commonly observed for transcription factors, but over a short time course. The cardinal feature of the circadian loop is that it takes approximately 24 hours to complete, because of the properties — presumably refined by natural selection — of its component elements. Mutations that affect these properties would alter the clock. For example, if the mutant proteins are more resistant to inactivation, the cycle will take longer to complete and the mutant's rhythms will run more slowly.

The molecular cycle can be entrained to solar time because some elements are sensitive to light. In *Neurospora*, light induces the expression of *frq* independently of any ongoing negative feedback. If the clock is running ahead of the solar cycle, later afternoon light will delay the spontaneous decline in *frq* expression and thereby delay entry to the next cycle, slowing down the dependent sporulation rhythms. Conversely, if the clock is lagging behind, light in the early morning will fall at a phase when mRNA levels are low. The light-induced expression of *frq* will accelerate the spontaneous increase in mRNA levels and thereby advance the clock and its dependent sporulation rhythms. The complex behaviour of the phase response curve can therefore be explained by relatively simple molecular events.

The effects of light on the *Drosophila* clock are more complex because feedback inhibition rests on interactions between the Per and Tim proteins, and light, acting via Tim, alters this interaction. But the principle is the same and, consistent with the autoregulatory model of circadian timing, circadian behaviour of transgenic flies and fungi *in vivo* can be reset by direct experimental manipulation of the expression of clock transgenes.

Clocks for the future

Molecular analysis of the clockwork is proceeding rapidly. How Per, Tim and Frq regulate their cognate genes and output genes driving adaptive rhythms is yet to be deciphered, but partnership with other transcriptional regulators is likely to play a significant part. The presence in Per of a PAS domain, a protein interaction sequence, has been a focus of attention, and recently PAS domains have been described in two other proteins encoded by clock-associated genes in *Neurospora* (*white collar 1* and *white collar 2*). The existence of PAS motifs in light-harvesting proteins has even prompted speculation on the evolution of clocks from photoreceptive mechanisms, an echo of the association between clocks and eyes.

But what about our own clock genes; do we have any? To date, two clock mutations have been described in mammals, the 20-hour clock of the *tau* hamster and the longer period, unstable clock of the *Clock* mouse. In a *tour de force* of forward genetic analysis, the mouse *Clock* gene has been cloned, sequenced and shown to be necessary for normal circadian function. It is expressed in the SCN and retina, as might be expected, along with several other tissues, and it too encodes a protein with a PAS domain.

The precise role of *Clock* awaits description, but what of *per*, which has been so well characterized in flies? A recent flurry of activity has led to some 'cross-over' success, first in the silk moth, where its mechanisms of action and contribution to clock functions present intriguing biological variations on the fruit fly theme. Now, an even bigger prize has been reported by two separate groups, with the cloning of *per* orthologues from human and mouse. The PAS sequence of *Drosophila per* provided the key to identifying these genes and, like *Clock*, the mouse forms of *per* (which are probably the same gene, named *rigui* by one of the groups) are expressed in the SCN. Moreover, and in contrast to *Clock*, levels of its transcript oscillate in the SCN with a 24-hour rhythm, and the phase of this SCN oscillation can be reset by switching the light–dark cycle. Is Rigui a 'clock component', an element of an auto-regulatory circadian feedback loop? In contrast to Period and Timeless, whose DNA-binding mechanisms are not understood, both Clock and Rigui have a basic helix–loop–helix DNA-binding motif which marks them as putative transcriptional regulators.

A further intriguing feature of *rigui* is that it is expressed in the *pars tuberalis* of the anterior pituitary. This overlooked gland has received some attention recently as a site mediating seasonal actions of melatonin. The reported expression of *rigui* in several melatonin-responsive tissues, the SCN, the retina and the *pars tuberalis*, has squared a circle between circadian clocks, seasonal timing and regulation by melatonin. We are now in a position to find out whether Clock and Per are partners in our clockwork, and how they are regulated by resetting cues. In that way we may discover how they conspire to get us to the lab on time, or not.

Acknowledgements

The author is extremely grateful to Dr Pat Lakin-Thomas and to Dr Andrew Millar for comments and advice on the manuscript. The author's work is supported by the Wellcome Trust and BBSRC.

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